The role of sister chromatid cohesion in cohesinopathies, DNA damage response and carcinogenesis

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2010

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CHAPTER

Chromatid cohesion defects in

human cancer cell lines

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ABSTRACT

Chromosomal instability is often associated with carcinogenesis and several mechanisms have been proposed that contribute to this phenotype. In colorectal cancer, defects in sister chromatid cohesion have been suggested to underlie chromosomal instability in a subset of tumors. Here, we confirm the occurrence of cohesion defects in 2 out of 12 colon carcinoma cell lines and show an even higher percentage (35%) in 17 head and neck squamous cell carcinoma (HNSCC) cell lines. In one of these cohesion-defective HNSCC cell lines, we found mutations in *PDS5A*, a gene encoding one of the proteins involved in sister chromatid cohesion. In another cell line, alternative splicing in *CHTF18* was found, which leads to exon 16 skipping and reduced levels of CHTF18, a protein known to be involved in chromatid cohesion. These data support the hypothesis that defects in sister chromatid cohesion may be mechanistically involved in generating genomic instability, which may play a causative role in tumorigenesis by contributing to aneuploidy.

INTRODUCTION

In human cancer, chromosomal instability results in loss or gain of whole chromosomes or parts of chromosomes, which characterizes most solid tumors. As a consequence, this instability leads to aneuploidy of the cancer cells. However, the connection between chromosomal instability and aneuploidy has not been defined clearly (reviewed in Holland & Cleveland 2009)[1]. Defects in sister chromatid cohesion have been described as a distinct mechanism involved in chromosomal instability, which was found in human colorectal cancer[2]. Mutations in a group of genes directly involved in sister chromatid cohesion were highly represented in 132 colorectal tumors with chromosomal instability. Sister chromatid cohesion is responsible for keeping sister chromatids together from DNA replication until cell division[3, 4]. The process is mediated by the cohesin complex, consisting of the subunits SMC1, SMC3, RAD21 (SCC1) and one of the two SA (SCC3) subunits, which together form a ring around the duplicated DNA to ensure proper cohesion. Regulatory proteins make sure the cohesin complex is loaded onto the DNA prior to DNA replication (NIPBL/SCC2 and MAU2/SCC4), establish cohesion at the replication fork (ESCO1, ESCO2) or have an 'antiestablishment-function' before replication and a cohesion maintenance function after replication (PDS5A, PDS5B, WAPL). Other proteins involved in sister chromatid cohesion e.g. CHTF18 and DDX11/ChlR1 have been implicated in the maintenance of replication fork stability in yeast[5, 6]. The mutations found in the chromosomal instable colon carcinoma samples were in three of the four core cohesin subunits, and in the *NIPBL* gene. These data indicate that defects in the process of sister chromatid cohesion may cause chromosomal instability in colorectal cancer. To further explore the role of sister chromatid cohesion in tumorigenesis, we investigated the presence of cohesion defects in colorectal cancer cell lines, and expanded the search to head and neck squamous cell carcinoma cell lines (HNSCCs). We found cohesion defects in colorectal carcinoma and HNSCC cell lines. This may point towards a more general role of sister chromatid cohesion defects in the development of human cancer.

RESULTS

Cohesion defects in colorectal cancer cell lines

A recent report suggested that sister chromatid cohesion defects may underlie chromosomal instability in a subset of colorectal cancers[2]. To further investigate this hypothesis, we examined a set of colorectal cancer cell lines for the presence of cohesion defects. In twelve cell lines examined, two cell lines displayed an increased amount of sister chromatid cohesion defects (for examples, see ref. 7). CaCo2 and Colo320, two

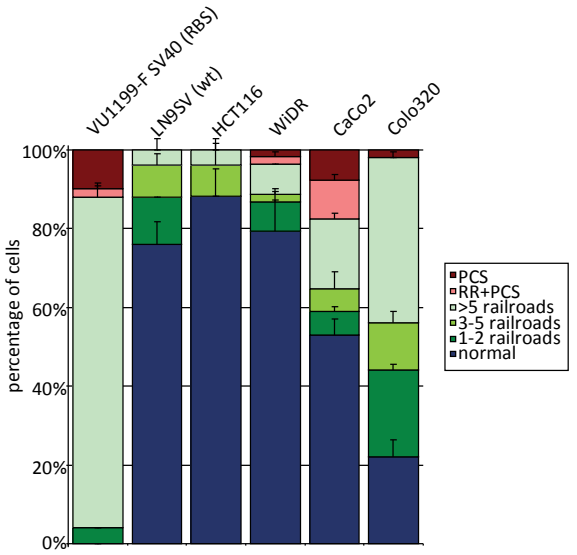

Figure 1. Cohesion defects in colon carcinoma cell lines. Metaphase spreads were examined for the presence of defects in sister chromatid cohesion, and percentages of cells with railroad chromosomes, railroad chromosomes plus total premature chromatid separation (PCS) or total PCS were scored.

chromosomal instable colon cancer cell lines, showed cohesion defects, especially railroad chromosomes, in 46-60% of the metaphases, while in the CaCo2 cell line also total loss of cohesion (premature chromatid separation, PCS) was observed (Figure 1). These levels of cohesion defects were not seen in immortalized wild type fibroblasts (LN9SV), nor in the microsatellite instable HCT116 or chromosomal instable WiDR colon cancer cell lines. The cell lines were screened for the expression of a set of proteins involved in sister chromatid cohesion, by Western blotting, but no abnormalities were found (data not shown). Although the molecular defects in these cancer cell lines remain to be determined, these data show that cohesion defects are present in a subset of colon carcinoma cell lines.

Cohesion defects in head and neck squamous cell carcinoma cell lines

In a panel of 17 head and neck squamous cell carcinoma (HNSCC) cell lines that we analyzed for mitomycin C-induced chromosomal breakage, cohesion defects were also observed. Quantification of railroad chromosomes and PCS in the metaphases of these cell lines revealed six cell lines with an increased incidence of cohesion defects (Figure 2). In cell lines 92VU041-T and UM-SCC-11B, around 40-50% of the cells showed cohesion defects, mainly railroad chromosomes. This was even more pronounced in cell line 92VU078-T, which displayed cohesion defects in over 95% of the cells. Intriguingly, these defects changed from railroads to total PCS in about 60% of the 92VU078-T cells by treatment with the DNA crosslinking agent mitomycin C (MMC), while in the other two cell lines the type of cohesion defects did not change. Interestingly, in cell line UM-SCC-22A, about 30% of the untreated cells had railroad chromosomes, which was induced up to 98% after the cells had been treated with MMC. DNA damage-induced cohesion defects

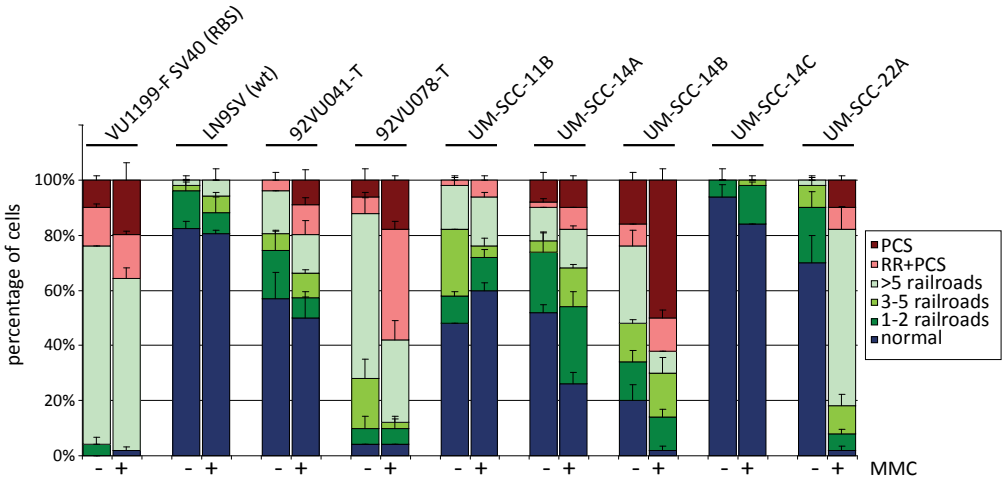

Figure 2. Cohesion defects in head and neck squamous cell carcinoma cell lines. Metaphase spreads were examined for the presence of defects in sister chromatid cohesion, and percentages of cells with railroad chromosomes, railroad chromosomes plus total premature chromatid separation (PCS) or total PCS were scored.

were also found in cell lines UM-SCC-14A and UM-SCC-14B, although these defects were also present in the untreated cells. The percentages of abnormal metaphases ranged from 50 to 98%, with the most severe defects observed in MMC-treated UM-SCC-14B cells. This cell line is derived from a local recurrence of the tumor from which cell line UM-SCC-14A was generated. The same patient developed a skin metastasis after chemotherapy; the cell line derived from this metastasis (UM-SCC-14C) did not show any cohesion defects. Together, these data reveal the presence of a wide diversity of cohesion defects in a relatively high proportion of HNSCC cell lines.

Mitomycin C sensitivity in cohesion-defective HNSCC cell lines

The cohesion-defective HNSCC cell lines were not particular sensitive for MMC in a chromosomal breakage assay, except for the cell lines that also showed damage-induced cohesion defects (92VU078-T, UM-SCC-14A, UM-SCC-14B and UM-SCC-22A, Figure 3a). These cell lines were the most sensitive for MMC in a growth inhibition test, though less sensitive than HNSCC cells from a Fanconi anemia patient (Figure 3b). Cell line 92VU078-T and UM-SCC-22A were also tested in a G2/M arrest assay and responded more sensitive to MMC when compared to the other tumor lines. In 92VU078-T there even seemed to be an elevated G2/M-phase peak in untreated cells, and after treatment a 8N peak appeared (data not shown). In contrast to the sensitivity by chromosomal breakage and growth

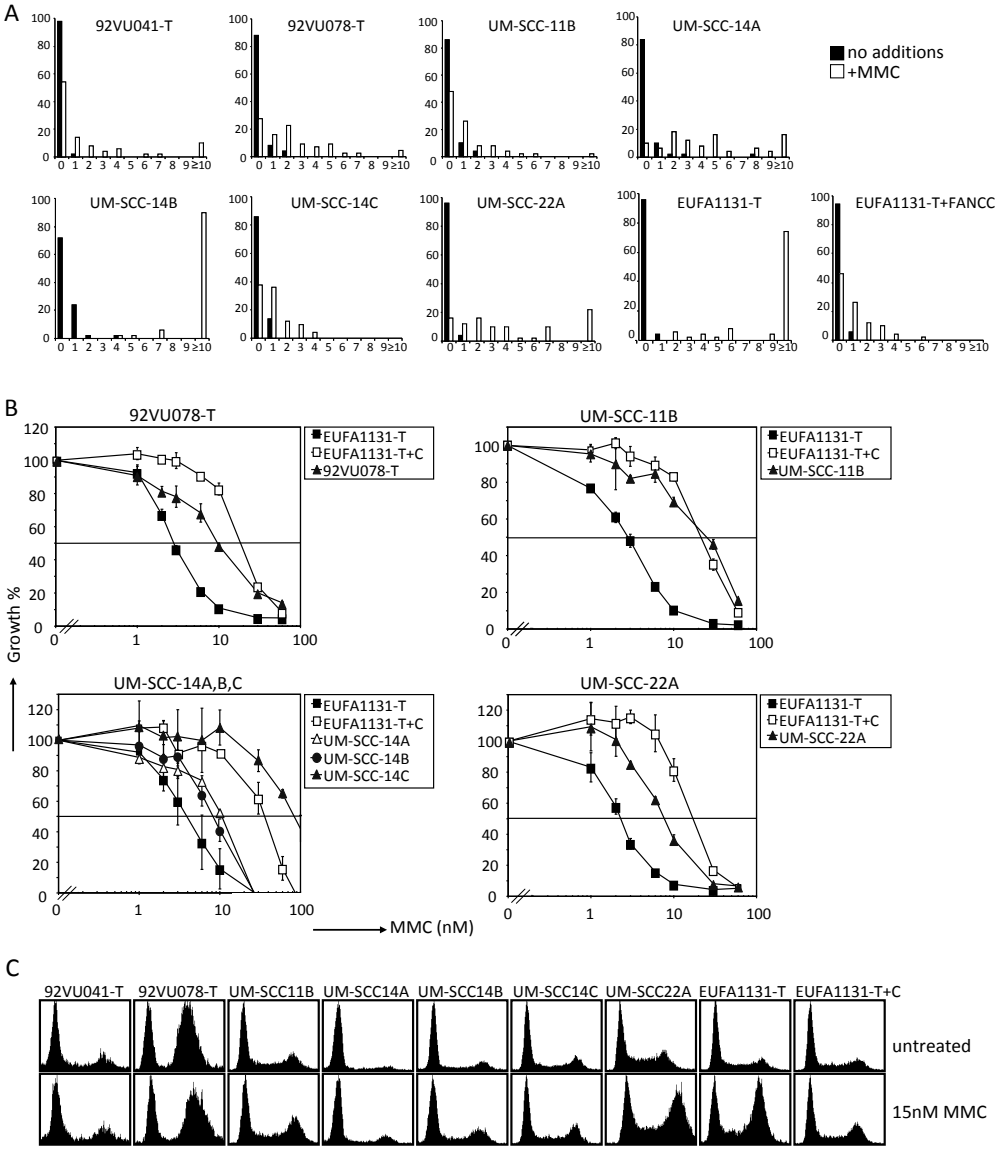

Figure 3. Mitomycin C sensitivity is correlated with damage-induced cohesion defects in head and neck squamous cell carcinoma cell lines (HNSCCs). (A) MMC-induced chromosomal breakage in HNSCC cell lines with cohesion defects. An HNSCC cell line from a MMC-hypersensitive Fanconi anemia C patient (EUFA1131-T) and its functionally corrected cell line (EUFA1131-T + FANCC) were used as controls. (B) MMC sensitivity in HNSCC as measured by growth inhibition. Sensitivity was compared to EUFA1131-T and EUFA1131-T + FANCC. (C) G2/M arrest after MMC treatment. Cells were treated with or without 15 nM MMC for 72 h, and cell cycle profiles were analyzed by FACS analysis.

inhibition, UM-SCC-14A and UM-SCC-14B cell lines did not show a G2/M arrest after MMC treatment, which might be explained by defective cell cycle checkpoint(s). Overall, these data indicate that damage-induced cohesion defects in HNSCC cell lines are correlated with their relative hypersensitivity to MMC.

DDX11, ESCO1, and ESCO2 in cohesion-defective HNSCC cell lines

Cohesion defects, either spontaneous or DNA-damage induced, are cellular phenotypes observed in Roberts syndrome and Warsaw breakage syndrome patients, which are caused by mutations in *ESCO2* and *DDX11*, respectively[7, 8]. The cellular phenotype of UM-SCC-14B and 92VU078-T, damage-induced PCS and MMC sensitivity, resembles that of cells from a Warsaw breakage syndrome patient. Therefore, DDX11 expression was investigated by Western blot analysis in extracts of the cohesion-defective HNSCC cell lines. DDX11 was detected in all of these cell lines, although the levels varied between the different samples (Figure 4). To further examine possible DDX11 defects, cDNA from UM-SCC-14B and 92VU078-T was sequenced and no abnormalities were found. These results exclude DDX11 deficiency in the cohesion-defective HNSCC cell lines.

The expression of ESCO2, and its related acetyltransferase ESCO1, was also analyzed by Western blot analysis in the cohesion-defective cell lines; both proteins appeared to be expressed at normal levels (Figure 4).

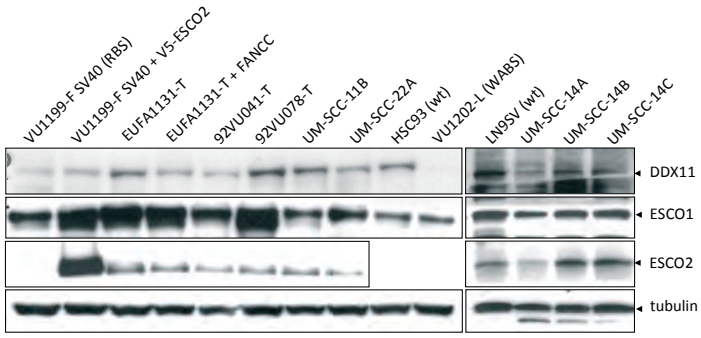

Figure 4. Expression levels of ESCO1, ESCO2 and DDX11 in HNSCC cell lines. Western blot analysis revealed normal expression of proteins defective in cohesinopathy syndromes.

92VU041-T is defective in cohesion protein PDS5A

In addition to DDX11, ESCO1, and ESCO2, the expression of a set of additional proteins known to be involved in sister chromatid cohesion was examined in all the HNSCC cell lines with cohesion defects. Western blotting showed the absence of PDS5A, a cohesin-complex associated protein, in cell line 92VU041-T (Figure 5a). This protein had a normal expression in fibroblasts from the same patient, which displayed no cohesion defects

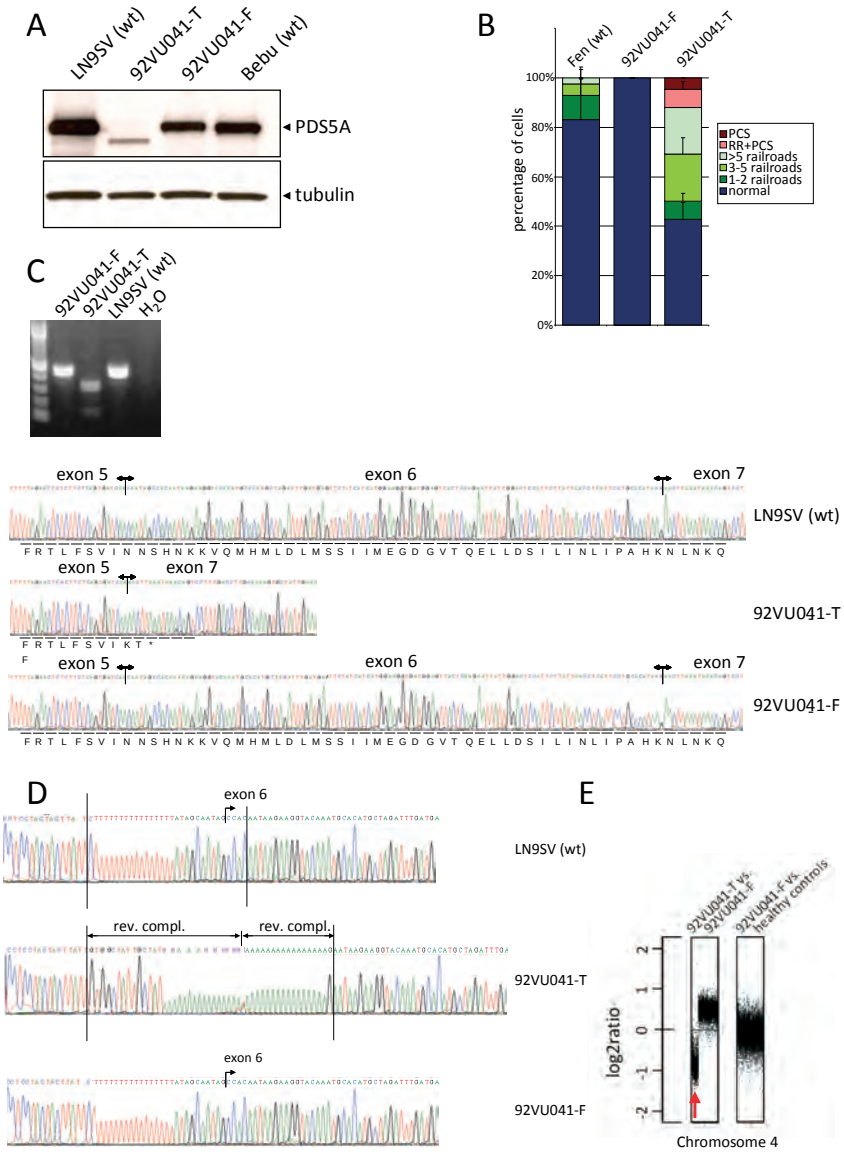

Figure 5. HNSCC cell line 92VU041-T is mutated in *PDS5A*. (A) Western blot analysis showing the absence of PDS5A protein in 92VU041-T and normal expression in fibroblasts of the same patient (92VU041-F). (B) Cohesion defects in 92VU041-T with 92VU041-F and wild type fibroblasts as a control. (C) Analysis of PCR products from cDNA with primers spanning from exon 3 to 7 on a 1% agarose gel showed a shortened PCR product, which was caused by the deletion of exon 6 from the cDNA. (D) Sequencing of genomic DNA revealed an inversion of a reverse complement sequence including a duplication at the start of exon 6 in 92-VU041-T. (E) Array comparative genomic hybridization of 92VU041-T versus 92VU041-F revealed loss of the p-arm of chromosome 4 in 92VU41-T (red arrow), while 92VU041-F cells are the same as normal controls (92VU041-F versus healthy controls).

(Figure 5b). PCR with cDNA primers spanning exon 3 to 7 detected a PCR product in 92VU041-T that was shorter than in wild type fibroblasts from the same patient (Figure 5c). The normal-sized PCR product was not found after 92VU041-T was treated with cycloheximide (data not shown). The shorter cDNA appeared to lack exon 6 sequence, resulting in a frame-shift and premature stop (Figure 5c). Further examination of the *PDS5A* gene revealed an inversion combined with a duplication of the inverted sequence at the intron-exon boundary of exon 6, which removes the splice-donor site (Figure 5d). This mutation was also found in paraffin-embedded tumor material (data not shown), indicating that it is not induced by culture conditions. In the tumor cell line, wild type PDS5A sequence was not detected, suggesting that the other *PDS5A* allele was deleted. Subsequent comparative genome hybridization (array CGH) indeed showed a heterozygous loss of the entire p-arm of chromosome 4, the region where the *PDS5A* is located (Figure 5e). This loss was not found in the control fibroblasts of the patient. Together these results strongly suggest that the cohesion defect observed in the 92VU041-T cell line is due to an acquired mutation in the *PDS5A* gene, accompanied by a loss of the wild type allele. Final proof should come from an experiment showing functional complementation of the defect by stable PDS5A cDNA expression; this experiment is currently pending.

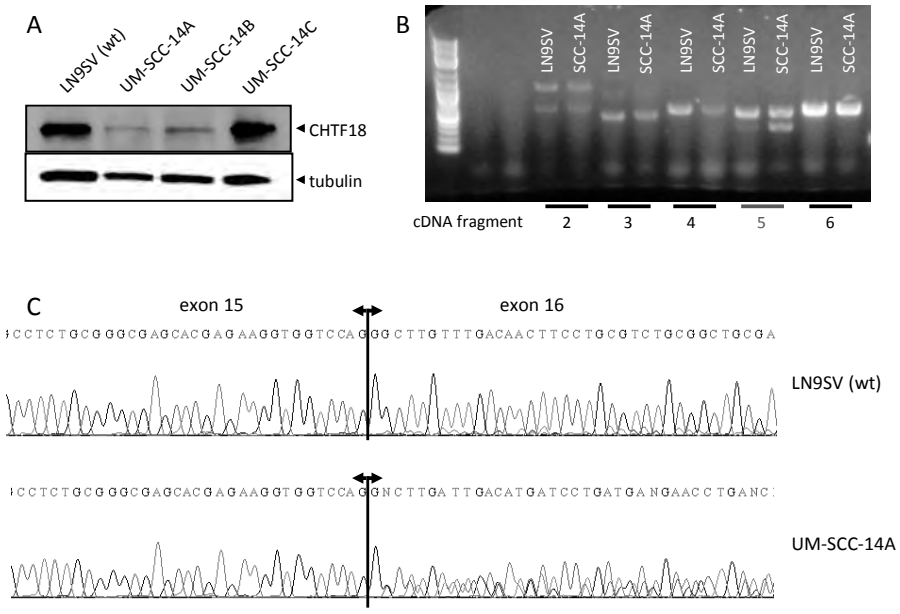

Figure 6. A splice-site mutation leads to reduced CHTF18 protein levels in UM-SCC-14A and UM-SCC-14B. (A) Western blot analysis on CHTF18 in UM-SCC-14 cell lines. LN9SV served as a wild type control. (B) Analysis of PCR products from cDNA with primers spanning from exon 15 to 16 (fragment 5) on a 1% agarose gel showed a shortened PCR product, which was caused by the deletion of exon 16 from the cDNA (C).

UM-SCC14A and UM-SCC14B have a defect in CHTF18

Although the cellular phenotypes of the UM-SCC14A and UM-SCC14B cell lines resemble that of the Warsaw breakage syndrome patient, no abnormalities were found in DDX11 protein expression. However, CHTF18, a protein that acts in close proximity of DDX11 at the replication fork, showed reduced levels when compared to UM-SCC-14C or wild type fibroblasts (Figure 6a). Subsequent sequence analysis on cDNA of UM-SCC-14A, UM-SCC-14B and UM-SCC-14C revealed a heterozygous deletion of exon 16 (Figure 6b). Though this deletion is in-frame, no truncated protein was detected by Western blotting with an antibody against the C-terminal part of the protein (Figure 6a).

DISCUSSION

In this study, we demonstrate that in colorectal carcinoma cell lines and HNSCC cell lines cohesion defects exist. Confirming the reported sequence variations in proteins involved in sister chromatid cohesion in colorectal tumors[2], cohesion defects were observed in two chromosomal instable colorectal cancer cell lines. In addition, we found even more frequent cohesion defects in cell lines derived from squamous cell carcinomas of the head and neck region. The cell lines showed cohesion defects in diverse patterns, such as MMC-induced defects, or the presence or absence of total PCS. These differences suggest that distinct mechanisms underlie the abnormalities of sister chromatid cohesion observed in these cell lines.

Sequence abnormalities in the *PDS5A* gene of cell line 92VU041-T led to a total lack of the protein, which is likely responsible for the partial cohesion defects seen in this cell line. This is in agreement with earlier findings showing that depletion of PDS5A by siRNA had mild effects on sister chromatid cohesion[9], likely due to redundancy in function with the related PDS5B protein. In the UM-SCC-14A and UM-SCC-14B cell lines, reduced CHTF18 protein levels due to alternative splicing were correlated with the observed cohesion defects. Although the UM-SCC-14C cell line also showed this mutation, it lacks cohesion defects. This might be due to an acquired compensatory mechanism in this metastasized cell line, e.g. amplification of the wild type allele. Increased cohesion defects after DNA damage were observed in several cohesion-defective cell lines.

Although we did not solve the molecular basis of the cohesion defects in all the cell lines, our study supports a correlation between the presence of cohesion defects and cancer. Whether this is a cause or a consequence for tumorigenesis remains to be clarified. Indications for the causative involvement of cohesion defects in carcinogenesis may be found when looking at the cohesinopathy syndromes. Though these syndromes are not established cancer predisposition syndromes, several observations indicate that

there is a connection. Some patients with Cornelia de Lange syndrome (CdLS), which is caused by a mutation in *SMC1A, SMC3* or *NIPBL*, developed metaplasia in the esophageal region. This suggests a genetic predisposition to esophageal alterations, although this is complicated because the disease is often accompanied by gastroesophageal reflux[10]. In addition, of the ~100 Roberts syndrome (RBS) cases described today, four patients were reported with neoplasia[11]. Considering that most RBS patient do not reach adulthood, this may point to an increased cancer risk for these cohesinopathy patients. In the family of the third cohesinopathy, Warsaw breakage syndrome (WABS)[7], a high cancer incidence is observed on mothers' side of the family, indicating that carriers of a *DDX11* mutation might be predisposed to cancer. Recently, also PDS5B has been implicated in tumor suppression[12]. Other indications for a role of sister chromatid cohesion in cancer is the finding that overexpression of the WAPL protein is correlated with cervical cancers[13, 14].

It is striking that a relatively high percentage of head and neck tumor cell lines presents with sister chromatid cohesion defects. In this type of tumors centromeric breakage and whole arm translocations have been noted to occur frequently[15-17], which may also apply to other tumor types[18-20]. These translocations may be facilitated by instability at the centromere due to cohesion defects, which are visible as railroad chromosomes. These data bring us closer to the idea that disruptions in sister chromatid cohesion may underlie the chromosomal instability which is a hallmark for many cancers.

MATERIALS AND METHODS

Ethics statement

The research on patient material was carried out after approval by the Institutional Review board of the VU Medical Center that adhered to local ethical standards, and was initiated only after the relevant informed consents had been obtained.

Cell culture

The SV40-immortalized RBS cell line (VU1199-F SV40) and wild type cell line LN9SV were cultured in Ham's F10 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA). Colon carcinoma (CaCo2, Colo320, WiDR, HCT116) and HNSCC (92VU041-T, 92VU078-T, UM-SCC-11B, UM-SCC-14A, UM-SCC-14B, UM-SCC-14C, UM-SCC-22A, EUFA1131-T, and EUFA1131-T+FANCC) tumor cell lines were cultured in DMEM (Gibco) supplemented with 10% FBS (Hyclone).

Western blot analysis

Whole-cell extracts were prepared in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, and 1% Triton X-100 supplemented with protease and phosphatase inhibitors). Proteins were separated on 8%-16% Tris-glycine gradient gels (Invitrogen, Eugene, USA) and transferred to Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked with 5% dry milk in TBST (10 mM Tris HCL pH 7.5, 150 mM NaCl, 0.05% Tween-20) and probed with rabbit anti-ESCO1 (1:1000, Bethyl laboratories, Montgomery, USA), purified guinea pig antiserum against $ESCO2_{217-359}$ (1:1000, described in ref. 21), mouse-anti DDX11 (1:1000, Abnova, Taipei city, Taiwan), rabbit-anti PDS5A (1:1000, Abcam, Cambridge, UK) mouse-anti CHTF18 (1:500, Abcam) or mouse-anti tubulin (1:20,000, Abcam). After washing with TBST, the membranes were incubated with peroxidase-conjugated goat immunoglobulins (DAKO, Glostrup, Denmark). Proteins were visualized with the ECL Western blotting detection system (Amersham Biosciences, Piscataway, USA).

Cohesion defects in metaphase spreads

Cells were grown to ~80% confluence, with or without exposure to 50 nM MMC (Kyowa Hakko Kogyo Co., Tokyo, Japan) for 48 hours. After 30 minutes demecolcin treatment (200 ng/ml, Sigma), cells were harvested, incubated with 0.075 M KCl for 20 minutes at room temperature, and fixed with 75% methanol, 25% acetic acid. Cells were dropped on a slide, air-dried, and stained for 5 minutes in a 4% Giemsa solution (Merck). For each culture, 50 metaphases were scored for the presence of cohesion defects, from coded slides. The percentages of metaphases with the indicated cohesion defect were determined.

Sequencing

PCR products were purified using a SAP/EXO treatment (Amersham Biosciences, Uppsala, Sweden) according to manufacturer's instructions. Sequencing reactions were prepared using specific primers and Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Samples were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Array CGH

Labeling and hybridization was done as previously described[22]. Briefly, 500 ng of genomic DNA of tumor and reference was labeled with either Cyanine 3-UTP (Cy3) or Cyanine 5-UTP (Cy5) nucleotide mixture, respectively, according to manufacturer's instructions (CGH labeling Kit for Oligo Arrays, Enzo Life Sciences, Farmingdale, NY, USA) and purfied using the QIAquick PCR Purfication Kit (Qiagen, Westburg, Leusden, The Netherlands). Labeled tumor and (matched) reference DNAs were mixed prior to hybridization onto Agilent 180

K oligonucleotide arrays (Agilent Technologies, Palo Alto, USA). Immediately after hybridization the slides were scanned using microarray scanner G2505B (Agilent technologies) and image analysis was performed using feature extraction software (version 9.1, Agilent Technologies). The Agilent CGH-v4_91 protocol was applied using default settings. Oligonucleotides were mapped according to the human genome build NCBI 6 (May 2006). Of both Cy3 and Cy5 channels, local ackground was subtracted from the median intensities. The log2 tumor to normal intensity ratio was calculated for each spot and normalized against the median of the ratios of all autosomes.

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